10. METABOLISM AND MECHANISM OF ACTION IN DIESEL EMISSION-INDUCED CARCINOGENESIS

Considerable research has been directed toward assessing the carcinogenic potential of diesel engine emissions. As indicated in Chapter 7, whole diesel exhaust (DE) is a pulmonary carcinogen in rats subjected to chronic exposures at high concentrations. This response to date has been clearly demonstrated only in rats, and apparently involves particle overload resulting in inflammation and proliferation of alveolar epithelial cells and subsequent tumor formation. Studies assessing diesel exhaust-induced carcinogenicity in hamsters were negative, and studies in mice were equivocal (depending on the strain). The organic components that are adsorbed to the diesel exhaust particle do not appear to be required for expression of the high-dose tumorigenic response in rats. Positive responses are also observed following exposure to carbon black (CB) and TiO_2 particles that lack the organic components. The organic components, however, are likely to play a greater role in tumorigenic responses at lower exposure concentrations. In susceptible mouse strains and in humans exposed at low concentrations, genotoxic mechanisms induced by the organic components may even play a predominant role. Epidemiologic data suggest that there is a small increased cancer risk in humans following long-term occupational exposure to diesel exhaust. In examining mechanisms of diesel exhaust-induced carcinogenicity, it is necessary to address several areas, including (1) the carcinogenic potential of the carbon particle and the particle-overload effect, (2) the metabolism and mechanism of action of known carcinogenic components such as benzo[*a*]pyrene (B[*a*]P) and various nitroarenes, (3) the role of pulmonary leukocytes, and (4) DNA adduct formation in diesel exhaust exposures.

10.1. PARTICLE-INDUCED CARCINOGENIC RESPONSE

DE is a pulmonary carcinogen in rats chronically exposed to high concentrations (Heinrich et al., 1986; Mauderly et al., 1987). Additional studies (Vostal, 1986; Kawabata et al., 1986; Heinrich, 1990; Wolff et al., 1990; Oberdörster and Yu, 1990) provided data indicating that the carbonaceous core was a major factor in this response. In addition to diesel exhaust particulate matter (DPM), particle-specific pulmonary carcinogenesis in rats has been demonstrated for particles with virtually no adsorbed organics, such as CB (Heinrich et al., 1994, 1995; Nikula et al., 1995) and particles with no organic component, such as TiO_2 (Lee et al., 1985, 1986; Heinrich et al., 1995). Results of studies (see Chapter 7) from both the Inhalation Toxicology Research Institute (Nikula et al., 1995) and the Fraunhofer Institute of Toxicology and Aerosol Research (Heinrich et al., 1995) have affirmed the instrumental role of the carbon core in producing a carcinogenic response in rats chronically exposed to high concentrations (>2 mg/m^3) of whole diesel exhaust. The results of these studies clearly indicate that a particle overload effect is

primarily responsible for this response. The rat alveolar epithelium may be predisposed to proliferative, metaplastic, and neoplastic responses, but the underlying mechanism for this effect is not clear. This section briefly reviews data affirming the particle effect, as well as data that provide some insight into possible mechanisms for this response.

A preliminary report by Heinrich (1990) provided evidence for a particle effect. In this study, female Wistar rats (72 per group) were exposed to Printex 90 CB particles for 10 mo followed by a 20-mo exposure-free observation period or for 20 months followed by a 10-mo exposure-free observation period. A particle concentration of 6.09 mg/m^3 was used in both protocols. The Printex 90 particles had an extremely low organic content (~1,000-fold less than that of DPM). The tumor rates for the 10- and 20-mo exposure durations were 17% (14% malignant) and 8% (all malignant), respectively. Although the lower tumor incidence for the longer exposure period was not consistent, the results demonstrate that the tumor incidences for CB particles with an organic content 1,000-fold less than DPM are equivalent to those reported for diesel exhaust exposures. The fact that these particles were able to exert a significant tumorigenic response implicates the carbon core of diesel exhaust particles as the possible tumor initiator in diesel exhaust-induced carcinogenicity at high particle concentrations.

More recently, an extensive study at the Fraunhofer Institute of Toxicology and Aerosol Research assessed the tumorigenic potential of DPM, the carbon core of DPM, CB, and TiO_2 in rats and two strains of mice (Heinrich et al., 1995). In this study, Wistar rats and NMRI mice were exposed to diesel engine whole exhaust, CB particles (Printex 90), and ultrafine TiO_2 particles for 2 years and to clean air for an additional 6 months. The results showed that when incidence of either all tumors or benign keratinizing cystic squamous-cell tumors was excluded, CB was at least as potent as DE when lung particle burdens were comparable (see Chapter 7 for details). DE was more potent on the basis of administered dose. The latter relationship can be at least partially explained by the slower pulmonary clearance of DPM and suggests that DE may be more toxic to phagocytic cells responsible for particle clearance.

Research efforts at the Inhalation Toxicology Research Institute (ITRI) have also provided data regarding the carcinogenic potential of whole diesel exhaust and of CB particles. In a long-term study, rats were exposed 16 h/day, 5 days/week for 24 mo to whole DE or CB (free of adsorbed organics) at particle concentrations of 2.5 or 6.5 mg/m^3 (Mauderly et al., 1991; Nikula et al., 1995). Controls were exposed to clean air. Although the CB particles were not totally devoid of organic components, the solvent-extractable fraction was small and the CB mutagenicity per unit of particle mass was three orders of magnitude less than that of diesel exhaust soot. Lung weights were increased in rats exposed to the highest concentrations of both diesel exhaust or CB but were slightly higher for the diesel exhaust group. The lung burdens of particulate matter were significantly greater for the diesel exhaust-exposed rats at 18 and 23 mo.

A substantial transfer of particles from the lungs to lung-associated lymph nodes was observed, but no difference was noted between the diesel exhaust and CB exposure groups. Inflammation and cytotoxicity detected in lavage fluid were greater for diesel exhaust-exposed rats, but the difference was proportional to the higher lung burden of retained particles noted for these animals. Tumor incidences and prevalence were similar for diesel soot-exposed and CB-exposed rats. Tumor types observed included squamous cysts, squamous cell carcinomas, papillary adenocarcinomas, tubular adenocarcinomas, and solid carcinomas. The growth of tumors transplanted into athymic mice also has been similar for diesel exhaust and CB exposures (74% and 73%, respectively).

Additional support for the particle-overload effect has been provided by data showing similar lung tumor rates in rats following intratracheal instillation of diesel exhaust soot or CB particles (Pott et al., 1994). Total primary lung tumors (expressed as percent and including adenomas, adenocarcinomas, cystic keratinizing squamous cell tumors, and squamous cell carcinomas) for the three DE groups were 65%, 60%, and 66%, whereas total tumors for the CB group were 65%. In addition to similar tumor rates, tumors induced by DPM and CB exhibited similar histopathologic patterns. Finally, Kawabata et al. (1993) induced lung tumors in rats intratracheally instilled with DPM from which the organic components had been extracted.

Wolff et al. (1990) reported the results of a study comparing pulmonary inflammation and DNA adduct formation in rats exposed 7 h/day, 5 days/week for 12 weeks to diesel exhaust or CB at concentrations of 10 mg/m^3. Although the level of lung DNA adducts was slightly higher for DE exposure, both exposures resulted in inflammatory responses, as determined by increased numbers of neutrophils and macrophages and increased acid proteinase in the bronchoalveolar lavage fluid.

Oberdörster and Yu (1990) evaluated the relationship between tumorigenic response of the lung and physical characteristics of various insoluble particles. Based on data from studies examining the effects of long-term inhalation exposure to DE TiO_2 particles, CB, or toner particles, they found that only the surface area of retained particles in the lung showed a reasonable concentration-response relationship relative to tumor incidence. Based upon this information, particle overload (retained mass or volume of particles) may not be the only determining factor in lung tumor formation for insoluble particles. The investigators hypothesized that a tumorigenic effect would probably require that a "critical" surface area of retained particles be attained for the manifestation of any mechanisms of tumorigenicity.

In comparing the potency of DE and CB, therefore, the particle surface area requires consideration. The Printex particles used in the study by Heinrich et al. (1995) have a large surface area ($\approx$227 m^2/g), whereas the surface area of DPM varies from 18 (unextracted organics) to 107 m^2/g (organics fully extracted). Although the actual mean surface area of DPM in the

lungs is uncertain, it is less than that of the Printex particles. It could be hypothesized that particle effects from diesel exposure are less than those of CB because of a smaller specific surface, but the difference in potency is made up by surface-associated organics. Similar conclusions might also be derived from the intratracheal studies reported by Pott et al. (1994). Proof for this hypothesis may be difficult to achieve because surface area of the diesel particle would be expected to increase as organics are eluted from the particle surface during residence time in the lungs.

The involvement of persistent alveolar epithelial hyperplasia appears to be associated with the induction of neoplasia by particles. Oberdörster et al. (1995) reported an inflammatory influx of neutrophils in rats exposed for 3 mo to CB at concentrations of 7 or 50 mg/m^3 but not at 1 mg/m^3. Data showing exposure-response relationships for cytokine expression, neutrophilic inflammation, and dose-dependent alveolar Type II epithelial cell mutagenesis in rat lungs following 13-week exposure to CB (7 or 50 mg/m^3) were reported by Driscoll et al. (1995). Although these effects are seen at lower exposure concentrations as particle size decreases and specific surface area increases, the effects of ultrafine particles at less than particle overload conditions is uncertain.

In summary, results of these studies indicate that little difference exists in the type or incidence of lung tumors in rats following long-term exposure to DE or CB at high concentrations, that particle-associated organics are likely to play a minor role in lung particle overload-induced pulmonary carcinogenicity of DE in rats, and that particle effects are clearly relevant to the carcinogenic response observed. Persistent inflammation and hyperplasia of the alveolar epithelium, cytokine release, and release of oxidant reactants by alveolar macrophages (see Section 10.3) appear to be key elements leading to the species-specific neoplastic condition. The mechanistic basis for the surface-area-associated effects on cancer potency is as yet unknown.

10.2. METABOLISM AND MECHANISM OF ACTION OF ORGANIC CARCINOGENIC COMPONENTS OF DIESEL EXHAUST

DE is a complex mixture containing gaseous-phase components as well as soot particles to which more than 450 organic compounds are adsorbed (Opresko et al., 1984; Nikula et al., 1995). Although the involvement of particle-adsorbed organics in pulmonary carcinogenic responses appears to be minor in rats under particle overload conditions, the contribution of a subtle direct-mutagen effect should not be summarily dismissed, especially for humans.

In other species, such an effect may be more relevant, although currently available animal data and epidemiologic data do not support such a contention. A direct mutagen effect may be inconsequential in rats relative to particle overload effects because bioavailability of the organic components is limited by the high-energy binding of these components with the carbon core,

resulting in low desorption in the biological environment. The long residence times of particles in human lungs, however, may allow for greater desorption of organics.

The mechanism of action of many PAH carcinogens has been attributed to the reactivity of certain metabolic intermediates with cellular macromolecules and the subsequent formation of DNA adducts. The organics adsorbed to DPM may become available for biotransformation to known reactive intermediates, and macromolecular binding of these metabolites has been demonstrated.

Except for some of the DNA adduct studies, the available database does not allow a definitive discussion of the specific mechanism of carcinogenic action for these compounds relative to diesel exhaust specifically but rather is approached from the standpoint of the chemicals per se. Some of the data are derived primarily from in vitro studies that were not specifically concerned with the potential carcinogenicity of diesel exhaust but may be relevant because the compounds of concern are known components of diesel emissions.

More than 100 carcinogenic or potentially carcinogenic components have been specifically identified in diesel emissions, including various PAHs and nitroarenes such as 1-nitropyrene (1-NP) and dinitropyrenes (DNPs). These compounds are adsorbed to the carbon core of the particulate phase of the exhaust and, if desorbed, may become available for biological processes such as metabolic activation to mutagens. Among compounds identified from diesel exhaust are B[*a*]P, dibenz[*a*,*h*]anthracene, pyrene, chrysene, and nitroarenes such as 1-NP, 1,3-DNP, 1,6-DNP, and 1,8-DNP, all of which are mutagenic, carcinogenic, or implicated as procarcinogens or cocarcinogens (Stenback et al., 1976; Weinstein and Troll, 1977; Thyssen et al., 1981; Pott and Stöber, 1983; Howard et al., 1983; Hirose et al., 1984; Nesnow et al., 1984; El-Bayoumy et al., 1988).

There is evidence supporting a carcinogenic role for organics in the combustion process. Mumford et al. (1989) reported greatly increased lung cancer mortality in Chinese communes burning so-called “smoky coal,” but not wood or smokeless coal, in unvented open-pit fires used for heating and cooking. Particle concentrations ranged from 10 to 25 mg/m^3 in communes burning either smoky coal or wood, but PAH levels were five to six times greater in the air of communes burning smoky coal. Thus cancer mortality correlated more closely with concentrations of PAHs than with particles. In the case of smokeless coal, both particle and PAH concentrations were low. Demonstration of the carcinogenicity of coke oven emissions in humans (Lloyd, 1971) also provided evidence for the role of organics, because coke oven particulate matter lacks an insoluble carbon core. It should be recognized, however, that PAH concentrations in these cases are much greater than can be expected from inhalation of DE.

Diesel particles may well enhance the activity of adsorbed organics. Adsorption of PAHs to a carrier particle such as hematite, CB, aluminum, or titanium dioxide enhances their

carcinogenic potency (Farrell and Davis, 1974). In a more recent report, adsorption to carbon particles greatly enhanced the tumorigenicity of pyrolyzed pitch condensate containing B[*a*]P and other aromatic carcinogens (Heinrich et al., 1995). The increased effectiveness can be partly explained by more efficient transport to the deep lung. Slow release also enhances residence time in the lungs and prevents overwhelming of activating pathways. Even though the organic constituents may be tightly bound to the particle surface, clearance half-times of nearly 1 year in humans (Bohning et al., 1982) allow time for elution to occur. Furthermore, Gerde et al. (1991) presented a model demonstrating that large aggregrates of inert dust containing crystalline PAHs are unlikely to form at doses typical of human exposure. This allows the particles to deposit and react with the surrounding lung medium, without interference from other particles. Particle-associated PAHs can then be expected to be released more rapidly from the particles. Bond et al. (1984) provided evidence that alveolar macrophages from beagle dogs metabolized B[*a*]P coated on diesel particles to proximate carcinogenic forms. Unless present on the particle surface, B[*a*]P is more likely to pass directly into the bloodstream and escape activation by phagocytic cells.

The importance of DE-associated PAHs in the induction of lung cancer in humans may be enhanced because of the possibility that the human lung is more sensitive to these compounds than rat lungs. Rosenkranz (1996) summarized information indicating that in humans and mice, large proportions of lung cancers contain both mutated *p*53 suppressor genes and K-*ras* genes. Induction of mutations in these genes by genotoxins, however, is much lower in rats than in humans or mice. It could be even be hypothesized that lung tumors in diesel-exposed mice, which apparently could be induced only when the animals were exposed from conception to increase sensitivity (Pepelko and Peirano, 1983), may be due primarily to the organic fraction.

10.2.1. Metabolism and Disposition of B[*a*]P Relative to Diesel Exhaust

It is generally recognized that B[*a*]P is an activation-dependent carcinogen, with the activated metabolites forming covalent DNA adducts (Boyland, 1980). The reactions responsible for this activation are mediated by the cytochrome P-450 monooxygenases and are known to occur in multiple tissues and in different species. The activation proceeds through Phase I oxidative and hydrolytic reactions, which result in the formation of the ultimate carcinogenic metabolite, B[*a*]P 7,8-dihydrodiol 9,10-epoxide. Specifically, B[*a*]P undergoes a mixed function oxidase (MFO)-mediated epoxidation to form B[*a*]P-7,8-oxide which, in turn, is subjected to an epoxide hydrolase-mediated hydrolysis resulting in the stereoisomeric diols (+)-B[*a*]P 7,8-dihydrodiol and (−)-B[*a*]P 7,8-dihydrodiol. The diasterioisomeric forms of B[*a*]P 7,8-diol 9,10-epoxide are derived following another P-450-mediated reaction.

Relative to DE carcinogenicity, several studies have examined the metabolism and disposition of constituents such as B[*a*]P. Mitchell (1982) subjected 24 male F344 rats to nose-

only inhalation of ^{3}H-B[*a*]P aerosol (500 mg/m^{3}) for 60 min. High levels of radiolabel were detected in the trachea, lungs, and turbinates. Based on measurement of the radiolabel, biphasic clearance was noted with half-time ($t_{1/2}$) values of 2 to 3 h and 25 to 56 h. Absorption by the lungs and systemic distribution were demonstrated by the presence of radiolabel in soft tissues, such as the liver, kidney, gastrointestinal tract, spleen, brain, and testes. The majority of the radiolabel in these tissues was removed after 2 days, and the major route of excretion was in the feces. The significance of this study is the demonstration of rapid absorption and systemic distribution of B[*a*]P and potential metabolites following inhalation exposure.

Metabolization of intratracheally instilled B[*a*]P (1.0 µg) in strain A/J mice exposed to diluted diesel exhaust (8 h/day, 7 days/week for 9 mo) was reported by Tyrer et al. (1981). The radiolabel (^{14}C or ^{3}H) was rapidly distributed throughout the body within 2 h. The highest levels were detected in the lungs, liver, and gastrointestinal tract. Only trace levels were detected in the gastrointestinal tract 168 h after administration. A companion study (Cantrell et al., 1980) examined the effects of prior DE exposure on in vivo B[*a*]P metabolism in the aforementioned mice. Homogenates of lung, liver, and testes were obtained from five mice sacrificed at 2, 24, or 168 h after B[*a*]P instillation. High-performance liquid chromatography (HPLC) analysis detected free B[*a*]P and nonconjugated primary metabolites and sulfate, glucuronide, and glutathione conjugates in each of the tissues. The occurrence of primary and secondary B[*a*]P metabolites in all three tissues was verified. The major hepatic metabolite was 3-hydroxy-B[*a*]P. The investigators concluded that diesel exhaust exposure may qualitatively affect the metabolism of B[*a*]P but does not significantly affect its distribution.

Sun et al. (1984) provided additional information comparing the disposition of particle-adsorbed B[*a*]P (0.1 wt %) and pure B[*a*]P following 30 min of nose-only inhalation by F344 rats. Long-term lung retention (percentage retained after 7 days) of particle-adsorbed ^{3}H-B[*a*]P was approximately 230-fold greater than that for pure ^{3}H-B[*a*]P. Pulmonary clearance of particle-associated ^{3}H was biphasic, with an initial $t_{½}$ of 1 h and a second-phase $t_{½}$ of 18 days, the latter representing clearance of 50% of the initially deposited radiolabel. Clearance of pure B[*a*]P aerosol was >99% within 2 h and was apparently caused by pulmonary and mucous membrane absorption into the blood rather than by mucociliary clearance and subsequent ingestion (Sun et al., 1982). Of the radiolabel retained in the lungs, 65% to 76% was B[*a*]P, 13% to 17% was B[*a*]P-phenol, and 5% to 18% was B[*a*]P-quinone. Although the Sun et al. (1984) study demonstrated the biotransformation of B[*a*]P to several metabolites, the epoxide intermediates known to be carcinogenic (Sims et al., 1974; Slaga et al., 1976) were not identified. However, B[*a*]P-phenol metabolites are reported to be mutagenic (Glatt and Oesch, 1976; Wislocki et al., 1986; Wood et al., 1976).

Leung et al. (1988) studied the role of microsomes in the removal and metabolism of B[*a*]P from DPM. Hepatic and lung microsomal preparations were made from 3-methylcholanthrene-induced F344 rats. ^{14}Carbon-B[*a*]P was adsorbed to DPM (0.49 μCi/mg) and incubated with the microsomal preparations. Results indicated that both lung and liver microsomes were capable of removing B[*a*]P from these modified exhaust particles and that this capacity was dependent on the lipid content of the microsomes. Only small (<3%) amounts of B[*a*]P were transferred from the particles, with only 1% to 2% of this being metabolized. Free B[*a*]P, however, was extensively metabolized by the microsomes to B[*a*]P-9-10-diol. Relative to the liver microsomes, the lung microsomes exhibited an approximate twofold greater efficiency in the transfer of particle-associated B[*a*]P.

Bond et al. (1984) demonstrated metabolism of particle-associated B[*a*]P and free B[*a*]P by alveolar macrophages (AMs). B[*a*]P-9,10-diol and B[*a*]P-7,8-diol were identified in the culture media, and B[*a*]P-7,8-diol and B[*a*]P-4,5-diol were detected in the cellular extracts. Additionally, small amounts of B[*a*]P phenols and B[*a*]P quinones were detected in both the cells and the media. The total amount of metabolites from both the cells and media increased with increasing incubation time up to 48 h. However, use of B[*a*]P in solution or B[*a*]P coated onto DPM did not alter the total amount of metabolites produced by the macrophages over a 24-h incubation period.

Because macrophages are instrumental in sequestering and transporting DPM matter in the lungs, AM-mediated metabolism of particle-associated B[*a*]P has been studied. Although some data show the ability of the AMs to metabolize B[*a*]P associated with DPM, Chen and Vostal (1982) have reported that aryl hydrocarbon hydroxylase (AHH) in AMs is decreased after in vivo exposure to diesel exhaust. Whether such diesel-associated decreases in AM enzymatic activity are counterbalanced by increases in the AM population size in response to diesel particle deposition (White and Garg, 1981) is unknown. Although it is known that human AMs contain AHH activity (McLemore et al., 1981) and that they can metabolize B[*a*]P (Harris, 1985), comparative studies of the AHH activities in rat, hamster, and human AMs could contribute toward determining the relationship such activity may have on the development of lung tumors.

Even though the AMs appear to contain the bulk of diesel particles deposited in the lung during chronic exposures, other cell types may also participate in the sequestration and/or metabolic activation of carcinogenic agents. The ability of lung epithelial cells to sequester diesel exhaust particles was reported by White and Garg (1981). Furthermore, significant metabolism of B[*a*]P by rat Type II alveolar epithelial cells was reported by Bond et al. (1983). In this study, a lung epithelial cell line (LEC) was shown to metabolize B[*a*]P to B[*a*]P-7,8-diol and B[*a*]P-9,10-diol, the latter accounting for 80% of the total B[*a*]P metabolites. Compared with the AMs that

were examined in the aforementioned study, the rat Type II cells showed approximately 10 times greater ability to metabolize B[*a*]P.

Under healthy conditions, the Type II cells represent about 12% to 16% of all cells in the pulmonary epithelium of mammalian lungs and account for approximately 4% to 9% of the cells in the lungs (Crapo et al., 1983). Alveolar macrophages, on the other hand, account for approximately 4% to 9% of the cells in the pulmonary region (Crapo et al., 1983). In terms of their relative abilities to metabolize B[*a*]P, the Type II cells may play an even more important role than the AMs in metabolically activating PAH, assuming PAH as a substrate is available to them (e.g., extraction of PAH from diesel particles by AMs and the subsequent release of PAH or metabolically susceptible metabolites of PAH at Type II cell sites). The Type II cell hyperplasia observed after the deposition of diesel and other types of particles (White and Garg, 1981; Lee et al., 1986; Lee et al., 1988; Plopper et al., 1983) seemingly would favor a prominent role for these cells in producing activated PAH metabolites.

Another cell type that may be important in the metabolism of PAH to ultimate carcinogens is the nonciliated bronchiolar cell. These cells are relatively rich in chemical metabolizing enzymes and, being also in a region of the respiratory tract where clearance of material would be relatively fast, may receive exposure via mucus to organics that have desorbed in the pulmonary region. The respiratory tract cytochrome P-450 system, for example, is present in Type II cells, but it is not as concentrated in this epithelial cell type as it is in the nonciliated bronchiolar cell (Boyd, 1984). It is worthy to note that bronchoalveolar adenomas that develop following diesel exposure have been found to resemble both Type II and nonciliated bronchiolar cells (Mauderly et al., 1987). Like the Type II cells, the nonciliated bronchiolar cells are not especially important in phagocytosis of particles deposited in the lung, although some particles may enter these cells. As previously indicated, any metabolism of procarcinogens by these cells probably involves the preextraction of carcinogen(s) in the extracellular lining fluid and/or in other endocytic cells.

Although the preceding studies indicate that particle-adsorbed B[*a*]P may be distributed throughout much of the organism via absorption from the lung and transport by the mucociliary escalator to the gastrointestinal tract, it is imperative to note that particles containing additional deposits of B[*a*]P exhibit greater potential for elution of organics than is observed for actual DPM. Therefore, the dissociation rates for the exhaust particles containing thermally deposited B[*a*]P do not realistically represent the dissociation of combustion-adsorbed organics. Even though particle-associated B[*a*]P can ultimately be metabolized by AMs and/or Type II cells to reactive intermediates, the contribution of this process to carcinogenic potential is uncertain and, in rats, is probably of questionable significance. The relevant importance may be different in humans, however, where particle clearance rates have half-times of ≈1 year, thereby allowing greater time for elution of organics.

10.2.2. Metabolism and Disposition of Nitroarenes

Diesel engine emissions contain a large number of components, including numerous nitroarenes. Quantitatively, the nitroarenes represent a relatively small contribution to the overall PAH component of diesel engine emissions. However, with respect to carcinogenic potential, some of the nitroarenes (e.g., 1-NP, 4-NP, 6-nitrochrysene, and some DNPs) are of concern because of their known or suspected carcinogenic activity and their high mutagenic activity in some test systems (Manabe et al., 1985; International Agency for Research on Cancer, 1989). Within the scope of this document, it is inappropriate to review all of the studies regarding the carcinogenicity, metabolism, and mechanism of action of these various nitroarenes. Therefore, emphasis has been placed on those nitroarenes considered by the International Agency for Research on Cancer (1989).

1-Nitropyrene, a genotoxic and carcinogenic nitrosubstituted organic, is a particle-associated component of diesel exhaust (Pitts et al., 1982; Schuetzle et al., 1982; King, 1988). As with B[*a*]P, several investigators have studied the metabolism and disposition of 1-NP both in free form and in association with DPM.

Bond and Mauderly (1984) made quantitative measurements of 1-NP metabolism and macromolecular covalent binding in the isolated perfused rat lung. The study verified oxidation, reduction, acetylation, and conjugation biotransformation of 1-NP by the lung, with oxidation being the major process. The major metabolites were 3-, 6-, and 8-hydroxynitropyrene. The overall metabolism of 1-NP was increased by prior exposure of the rats to the mixed-function oxygenase (MFO) inducer 3-methylcholanthrene (3-MC) but not to phenobarbital. This 3-MC-induced increase in 1-NP metabolism and a parallel increase in macromolecular covalent binding suggest that this pathway may be responsible for the observed covalent binding.

Exposure of rats to diesel exhaust (7.4 mg/m^3) for 7 h/day, 5 days/week for 4 weeks resulted in twofold increases in the rates of nitropyrene metabolism in nasal tissue and in isolated perfused lungs from these animals (Bond et al., 1986). HPLC analysis of ethyl acetate-extractable 1-[^{14}C]NP metabolites indicated that the major metabolites were 3-, 6-, and 8-hydroxy-1-aminopyrene and 4,5-dihydro-4,5-dihydroxy-1-nitropyrene. Furthermore, a fourfold increase in ^{14}C covalently bound in the lungs of these rats was detected. The increase in 1-NP metabolism was not observed for rats among lower exposure (0.35 or 3.3 mg/m^3) groups or clean air controls. The data from this study indicate that exposure to DPM matter at concentrations of 7.4 mg/m^3 significantly alters the metabolism and subsequent covalent binding of nitropyrene.

Bond et al. (1986) also examined the metabolism and deposition of free and particle-associated 1-NP in F344 rats. Results of the work indicated that the urinary and fecal excretion of ^{14}C-1-NP was not altered by exposure to the pure form or to that adsorbed on DPM. Pure 1-NP was more efficiently absorbed in the lung than was 1-NP coated onto DPM and, therefore,

greater lung retention was noted for particle-adsorbed 1-NP. However, no significant difference between the two forms of 1-NP was noted for extrapulmonary tissue distribution or metabolic profiles. Analysis of excreta and tissues indicated that 1-NP is rapidly metabolized by the lungs or metabolized by other tissues following translocation from the lungs. For both 1-NP forms, small amounts of 6- and 8- hydroxyacetylaminopyrene were detected in the lungs, suggesting pulmonary oxidation, reduction, and conjugation of the parent compound. The demonstration of pulmonary metabolism of 1-NP and greater retention of 1-NP when adsorbed to DPM may be significant relative to the dose to the lungs of both parent compound and metabolites.

Ball and King (1985) administered [^{14}C]1-NP to rats intraperitoneally, orally, or by intratracheal instillation of vapor-phase-coated diesel exhaust particles (380 µg [^{14}C]1-NP/g; 5 mg/rat). More than 50% of the radiolabel was recovered (20% to 30% in the urine and 40% to 60% in the feces) within 24 h, regardless of the route of administration. The metabolic profile and elimination kinetics were similar for all routes of administration. The principal urinary metabolite (representing 15% to 25% of the total urinary ^{14}C) was 6-hydroxy-*N*-acetyl-1-aminopyrene (6-OH-NAAP), a compound with demonstrated S-9-dependent mutagenic activity in *Salmonella* strain TA98. Gut flora was shown to be necessary for the formation of 6-OH-NAAP, for the observed enterohepatic circulation of metabolites excreted in the bile, and for excretion of mutagenic activity in the urine. That intestinal microorganisms may alter the metabolites of 1-NP and facilitate their reabsorption was also reported by Medinsky et al. (1985). Accumulation of ^{14}C and diesel exhaust particles was detected in the lungs and gastrointestinal tract 24 h after intratracheal administration, thereby attesting to the importance of mucociliary transport and distribution of particles and their adsorbed components. Based on these results and previous in vitro studies (King et al., 1983) demonstrating 1-NP binding to macromolecules, the authors note the possible risk to the gastrointestinal tract and lungs relative to 1-NP.

Howard et al. (1986) studied the binding of intratracheally instilled nitropyrenes and B[*a*]P to mouse lung DNA following preexposure to intratracheally instilled doses of the putative inducing agents B[*a*]P, dichloromethane extract of DPM, or 1-NP. The results indicated that 1-NP was a potent DNA-binding agent even in the absence of enzyme induction and that this potency was increased following B[*a*]P exposure. Dinitropyrene (a mixture of the 1,3-, 1,6-, and 1,8- isomers) was also a potent lung DNA-binding agent, with and without the inducers. Benzo[*a*]pyrene was not as potent a binding agent. Preexposure to the DPM extract but not to B[*a*]P resulted in increased DNA binding of B[*a*]P. Pretreatment with the dichloromethane extract of DPM failed to increase the DNA binding of the nitropyrenes. The significance of this report is the demonstration that exposure to DE may potentiate the DNA binding of some of its components.

The preceding studies have shown that some of the nitroarenes known to be constituents of DE may undergo biotransformation to various metabolites, some of which are known to be carcinogenic to animal species. Such data may become more relevant as a complete understanding is obtained regarding the desorption of these compounds from DPM and their subsequent availability for biotransformation processes.

Nitroarenes quantitatively represent a relatively small portion of the PAH component of DE and, at least in rats, may play a minor role in tumorigenic responses compared to the particle overload effect. However, their contribution to the potential carcinogenicity of diesel engine emissions deserves some consideration. In the previous section, information was presented regarding the in vivo and in vitro metabolism of various nitroarenes considered to be possible human carcinogens. The fact that some of these metabolites have been shown to form DNA adducts in animal studies and are mutagenic in several test systems warrants their inclusion in assessing possible mechanisms of diesel-exhaust-induced carcinogenicity. In fact, Gallagher et al. (1994) reported results suggesting that DNA adducts are formed from nitro-PAHs present in DE and may play a role in the carcinogenic process.

However, the question remains as to why animals exposed to DE do not develop tumors characteristic of those induced by dinitropyrene. Rosenkranz (1995, 1996) provided evidence in support of a hypothesis that highly carcinogenic dinitropyrenes present on DE are bioactivated only at low exposure concentrations. Specifically, diesel exhaust exposure-mediated oxidative stress may prevent the reduction of dinitropyrenes to arylhydroxylamines and the subsequent formation of reactive arylnitrenium species that form ^{8}C-DNA adducts. Such an oxidative stress may also result in the oxidation of the arylhydroxylamines to nitrosoarenes that are incapable of reacting with DNA (Boldt et al., 1991). Additionally, the inflammatory response resulting from particle overload also may induce oxidative stress-like conditions. This hypothesis suggests that there are other potential carcinogenic mechanisms that could be expressed in other species or under different exposure conditions.

10.2.3. Formation of Reactive Oxygen Species From Organic Constituents of Diesel Exhaust and Their Involvement in the Induction of Lung Cancer

Sagai et al. (1993) reported that diesel exhaust particulate matter (DEP) could produce superoxide and hydroxyl radicals without any biological activating systems. DEP washed with methanol could no longer produce these radicals, indicating that the active components were extractable with organic solvents. The likely involvement of reactive oxygen species in the induction of toxic and potentially carcinogenic effects in the lungs was suggested by greatly reduced lung injury due to DEP following extraction of the organic fraction prior to intratracheal instillation in mice. Additional support for the involvement of radicals in tissue damage was also

provided by the finding that pretreatment with superoxide dismutase (SOD), an antioxidant, markedly reduced lung injury and death due to instillation of DEP. Similarly Hirafuji et al. (1995) found that catalase, deferoxamine, and MK-447 inhibited the toxic effects of DEP on guinea pig tracheal cells and tissues in vitro. Nagashima et al. (1995) demonstrated that the production of 8-hydroxydeoxyguanosine (8-OHdG), a product of the reaction of guanine with oxygen radicals, is induced in mouse lungs by intratracheal instillation of DEP. Ichinose et al. (1997) reported further that while intratracheal instillation of washed DEP in mice induced a small but statistically significant increase in lung tumor incidence, unwashed DEP induced a larger increase. Moreover, increases in 8-OHdG correlated very well with increases in tumor rates. It thus appears likely that generation of reactive oxygen species resulting from exposure to DEP may be involved in the carcinogenic process. It should be noted, however, that this nucleoside is efficiently repaired and that proof of a causal relationship in humans is still lacking. It is also unlikely that superoxide or hydroxyl radicals chemically generated by DEP alone promote 8-OHdG production in vivo and induce lung toxicity, because SOD is extensively located in mammalian tissues. A recent study indicates that the major route by which active oxygen species are generated from DEP components is via the P450 reductase system (Kumagai et al., 1997).

10.3. POTENTIAL INVOLVEMENT OF PULMONARY LEUKOCYTES IN THE DEVELOPMENT OF LUNG TUMORS

Phagocytic leukocytes have been shown by numerous investigators to be toxic to tumor cells in vivo, and increasing evidence suggests that cells of the mononuclear phagocyte series in particular may be of pivotal importance in providing protection against malignancy in situ. This protective function may, at least in part, result from these cells' ability to produce a tumor necrosis factor (TNF) (Urban et al., 1986). Whether the tumor surveillance and tumoricidal activities of AMs (Hengst et al., 1978; Sone et al., 1983; Sone, 1986; Kan-Mitchell et al., 1985) are compromised or otherwise modified when they are engorged with even relatively benign particles has not been experimentally evaluated. The possibility remains that diesel and other types of particles at high lung burdens result in decreases in natural killer (NK) cell functional activities in providing defense against tumor formation, either by direct particle-cell interactions or by altering the ability of AMs to influence NK cell-mediated host defense against metastatic tumor cells (Sone, 1986). These cells are subpopulations of lymphocytes that possess spontaneous cytolytic activity toward neoplastic cells but not toward normal cells. Moreover, the tumoricidal function of cytotoxic T lymphocytes (Sone, 1986) may be directly or indirectly compromised by the presence of high lung burdens of particles in the lungs.

Phagocytes from a variety of species produce elevated levels of oxidant reactants in response to challenges such as phagocytic stimuli, with the physicochemical characteristics of a

phagocytized particle being a major factor in determining the magnitude of the oxidant-producing response. Hatch and co-workers (1980) have demonstrated that interactions of guinea pig AMs with a wide variety of particles, such as silica, metal oxide-coated fly ash, polymethylmethacrylate beads, chrysotile asbestos, fugitive dusts, polybead carboxylate microspheres, glass and latex beads, uncoated fly ash, and fiberglass increase the production of reactive oxygen species. Similar findings have been reported by numerous investigators for human, rabbit, mouse, and guinea pig AMs (Drath and Karnovsky, 1975; Allen and Loose, 1976; Beall et al., 1977; Lowrie and Aber, 1977; Miles et al., 1977; Rister and Baehner, 1977; Hoidal et al., 1978). As well, polymorphonuclear leukocytes (PMNs) are also known to increase production of superoxide radicals, hydrogen peroxide, and hydroxyl radicals in response to membrane-reactive agents and particles (Goldstein et al., 1975; Weiss et al., 1978; Root and Metcalf, 1977).

It is well recognized that the deposition of particles in the lung can result in the efflux of PMNs from the vascular compartment into the alveolar space compartment in addition to expanding the AM population size. Following acute exposures, the influx of the PMNs is transient, lasting only a few days (Adamson and Bowden, 1978; Bowden and Adamson, 1978; Lehnert et al., 1988). Strom (1984) has reported that PMNs become abnormally abundant following chronic exposures to DE. In the study by Strom (1984), the numbers of PMNs lavaged from the lungs of diesel-exposed rats generally increased with increasing exposure duration and inhaled DPM concentration. Strom (1984) also found that PMNs in diesel-exposed lungs remained persistently elevated for at least 4 mo after cessation of exposure, a potential mechanism that may be related to an ongoing release of previously phagocytized particles by AMs that engulfed them shortly after deposition. Evidence in support of this possibility has been obtained by Lehnert et al. (1989) in a study in which rats were intratracheally instilled with 0.85, 1.06, or 3.6 mg of polystyrene particles. The PMNs were not found to be abnormally abundant during the clearance of the two lower lung burdens, but they did become progressively elevated in the lungs of the animals in which alveolar-phase clearance was restored. Moreover, the particle burdens in the PMNs became progressively greater over time. Such findings are consistent with an ongoing particle relapse process, given the relatively short lifespan of PMNs. As previously indicated, lung tumors develop in the rat at lesser lung burdens of DPM than with particles such as TiO_2. Polymorphonuclear leukocytes characteristically are increased abnormally in the lung by DE exposure, but their presence in the lungs does not appear to be excessive following the pulmonary deposition of even high lung burdens of TiO_2 (Strom, 1984; Lee et al., 1986). Thus, the generation of reactive oxygen species by both AMs and PMNs should be considered as one potential factor of what probably is a multistep process that culminates in the development of lung tumors in response to chronic deposition of DPM.

As previously indicated, the production of oxygen species may afford protection against emerging tumor cells by killing the cells, whereas under other conditions the production of reactive oxygen products conceivably may contribute to the development of neoplastic cells. The potential involvement of AMs and PMNs in the development of lung tumors in laboratory rats administered high lung burdens of DPM (Mauderly et al., 1987) or having inhaled particles that are generally considered to have low to no cytotoxic potential (e.g., TiO_2 [Lee et al., 1986]) over a prolonged period of time may be related to the ability of the lung-free cells to produce reactive oxygen metabolites during phagocytic oxidative metabolism (Hatch et al., 1980). Even though products of phagocytic oxidative metabolism, including superoxide anions, hydrogen peroxide, and hydroxyl radicals, can kill tumor cells (Klebanoff and Clark, 1978), and the reactive oxygen species can peroxidize lipids to produce cytotoxic metabolites such as malonyldialdehyde, some products of oxidative metabolism apparently can also interact with DNA to produce mutations. Along this line, Weitzman and Stossel (1981) found that human peripheral leukocytes were mutagenic in the Ames assay. This mutagenic activity was related to PMNs and blood monocytes; blood lymphocytes alone were not mutagenic. These investigators speculated that the mutagenic activity of the phagocytes was a result of their ability to produce reactive oxygen metabolites, inasmuch as blood leukocytes from a patient with chronic granulomatous diseases, in which neutrophils have a defect in the NADPH oxidase generating system (Klebanoff and Clark, 1978), were less effective in producing mutations than were normal leukocytes. Of related significance, Phillips et al. (1984) demonstrated that the incubation of Chinese hamster ovary cells with xanthine plus xanthine oxidase (a system for enzymatically generating active oxygen species) resulted in genetic damage hallmarked by extensive chromosomal breakage and sister chromatid exchange and produced an increase in the frequency of thioguanidine-resistant cells (HGPRT test). Aside from interactions of oxygen species with DNA, increasing evidence also points to an important role of phagocyte-derived oxidants and/or oxidant products in the metabolic activation of procarcinogens to their ultimate carcinogenic form (Kensler et al., 1987).

Another characteristic of AMs and PMNs that may contribute to the pathologic process leading to lung tumor development following DPM deposition is that these phagocytes are known to release a variety of potentially destructive hydrolytic enzymes, a process known to occur simultaneously with the phagocytosis of particles (Sandusky et al., 1977). The essentially continual release of such enzymes during chronic particle deposition and phagocytosis in the lung may be detrimental to the alveolar epithelium, especially to Type I cells. Evans et al. (1986) showed that injury to Type I cells is followed shortly thereafter by a proliferation of Type II cells. Type II cell hyperplasia is a generally common feature observed in the lungs of animals that have received high lung burdens of various types of particles, including unreactive polystyrene microspheres. Exaggerated proliferation as a repair or defensive response to DPM deposition

may have the effect of amplifying the likelihood of neoplastic transformation in the presence of carcinogens beyond that which would normally occur with lower rates of proliferation, assuming an increase in the cell cycling of target cells and the probability of a neoplastic-associated genomic disturbance.

The proliferative response of Type II cells following the deposition of DPM or other types of particles, however, has yet to be directly related to a Type I cell destruction by proteolytic enzymes released by lung phagocytes or to a direct action of particles on the proliferation kinetics of the Type II cells. The production of reactive oxygen species or their products could also be involved in the process. Whatever the stimulus, it remains possible that the lung's AM population may play a role aside from any responsibility for Type I cell damage. Alveolar macrophages have the ability to release several other effector molecules or cytokines that can regulate numerous functions of other lung cells, including their rates of proliferation (Bitterman et al., 1983; Jordana et al., 1988; Denholm and Phan, 1989). The AM-derived mediators that may stimulate Type II cell hyperplasia following particle deposition in the lung remain to be identified, if in fact the AMs play a regulatory role in the Type II cell proliferative response.

Driscoll (1995) and Oberdörster and Yu (1991) outlined a proposed mechanism for the carcinogenicity of DE at high doses that emphasizes the role of phagocytic cells. Following exposure, phagocytosis of particles acts as a stimulant for oxidant production and inflammatory cytokine release by lung phagocytes. It was hypothesized that at high particle exposure concentrations the quantity of mediators released by particle-stimulated phagocytes exceeds the inflammatory defenses of the lung (e.g., antioxidants, oxidant metabolizing enzymes, protease inhibitors, cytokine inhibitors, etc.), resulting in tissue injury and inflammation. With continued particle exposure and/or the persistence of excessive particle burdens, there then develops an environment of phagocytic activation, excessive mediator release-tissue injury and, consequently, more tissue injury, inflammation, and tissue release. This is accompanied by cell proliferation. As discussed in a review by Cohen and Ellwein (1991), conceptually cell proliferation can increase the likelihood that any oxidant-induced or spontaneously occurring genetic damage becomes fixed in a dividing cell and is clonally expanded. The net result of chronic particle exposures sufficient to elicit inflammation and cell proliferation in the rat lung is an increased probability that the genetic changes necessary for neoplastic transformation will occur. In support of this hypothesis, it was reported that concentrations of inhaled CB resulting in lung inflammation increased mutation rates, an effect ameliorated by treatment with antioxidants (Driscoll, 1995). Although these responses can occur in the absence of organic carcinogens, those present in DE may still contribute to the process.

The possibility that particles induce carcinogenic effects only at high doses still lacks conclusive proof. For example, it has not been definitively shown that inflammatory responses are

a prerequisite for tumor induction. Furthermore, direct effects of ultrafine particles such as DPM taken up by epithelial cells cannot be ruled out. In fact, Riebe-Imre et al. (1994) reported that CB is taken up by lung epithelial cells in vitro, inducing chromosomal damage and disruption of the cytoskeleton, lesions that closely resemble those present in tumor cells. Johnson et al. (1993) reported that 20 nm polytetrafluoroethylene particles are taken up by pulmonary epithelial cells as well as polymorphonuclear leucocytes, inducing an approximate 4-, 8-, and 40-fold increase in the release of interleukin-1 alpha and beta, inducible nitric oxide synthetase, and macrophage inflammatory protein, respectively.

10.4. DNA ADDUCT CONSIDERATIONS

Although DNA adduct formation by particle-adsorbed organics was originally thought to be a mechanistic component of the diesel exhaust-induced pulmonary carcinogenesis observed in rats, currently available data do not support this hypothesis. The following section provides a brief overview of studies investigating DNA adduct formation following exposure to PAHs, DE, and other particulate matter.

On the assumption that DNA adduct formation is a critical step in the initiation of carcinogenesis (Harris, 1985), it was hypothesized that increased residence time of PAHs in the lung would increase the opportunity for metabolism and subsequent adduct formation. This would be especially important if association of the PAHs with soot particles and their slow release from those particles contributed to the increased residence time. Therefore, adduct formation by B[*a*]P alone compared with that of particle-associated B[*a*]P was investigated regarding possible mechanisms of diesel exhaust carcinogenicity.

An experiment was undertaken to test the hypothesis that inhalation of B[*a*]P associated with CB particles would increase the levels of DNA adducts compared with inhalation of pure B[*a*]P (Wolff et al., 1989). DNA modification was measured using the ^{32}P-postlabeling method developed by Randerath et al. (1985). The high sensitivity (~1 adduct in 10^{10} bases) of this technique (Reddy and Randerath, 1986) made possible measurement of the low levels of DNA adducts resulting from repeated inhalation exposures to ^{14}C-B[*a*]P aerosols (2 mg/m^3), ^{14}C-B[*a*]P (2 mg/m^3) adsorbed to CB particles (97 mg/m^3) (B[*a*]P/CB), or filtered air. Total ^{14}C levels in the lung (a nonspecific indicator of reactive and nonreactive B[*a*]P metabolites, free B[*a*]P, and particle-bound B[*a*]P) were 100-fold greater following exposure to B[*a*]P/CB than following exposure to B[*a*]P alone.

The levels of total DNA adducts or the B[*a*]P diol-epoxide(BPDE)-DNA adduct in the lung were *not* significantly different whether the rats were exposed to pure B[*a*]P or B[*a*]P/CB. However, association of B[*a*]P with CB resulted in the formation of unidentified lung adducts that were not seen in DNA from lungs of rats exposed to pure B[*a*]P. It is possible that the adducts

seen only in the B[*a*]P/CB exposures may play a role in the potential tumorigenic effect of particle-associated B[*a*]P. Reasons for the discrepancy between particle effects on total DNA adducts and retention of ^{14}C include the possibility that the kinetics for formation and decline of DNA adducts are different from those of total bound ^{14}C. As a consequence, long-term retention of total B[*a*]P and metabolites in the lung may not be a good marker for adduct formation.

There were clear differences in the kinetics of the buildup and decline of DNA adduct levels and total ^{14}C for rats exposed to B[*a*]P/CB. The $t_{1/2}$ for the decline of total ^{14}C was approximately 10-fold faster than that for the decline in levels of DNA adducts for rats exposed to B[*a*]P/CB. Previous work has shown that at 1 day or later after the end of single exposures to B[*a*]P or B[*a*]P/CB, most of the ^{14}C present was bound to total macromolecules (Sun et al., 1988), presumably largely non-DNA protein. This information in combination with the current data suggests that decline or repair of DNA adducts is considerably faster than that of protein turnover. Following repeated exposures, this would be expected to lead to increased buildup of ^{14}C in the lung relative to DNA adducts. The $t_{1/2}$ values for decline in DNA adducts observed in the current work are similar to the $t_{1/2}$ values of approximately 4 weeks reported for B[*a*]P metabolite-DNA adducts in the lungs of A/HeJ and C57BL/6J mice (Stowers and Anderson, 1985). Protein turnover is generally on longer time scales than the aforementioned $t_{1/2}$ values.

It appears that long-term retention of ^{14}C radiolabel in the lung may not be as important as previously suspected, at least with respect to indicating DNA damage. ^{14}C binding levels and DNA adducts were not closely related, and it is clear from these results that DNA adduct levels cannot be predicted from total ^{14}C levels. This observation is consistent with the work of Morse and Carlson (1985), who observed that binding levels of ^{3}H with lung protein were greater than levels of ^{3}H to lung DNA 6 h after administration of oral H-B[*a*]P to mice. They also found that ^{3}H binding to protein was more persistent than ^{3}H binding to DNA.

Caution should be used in interpreting the results from short-term exposures in regard to possible implications for long-term exposures when carcinogenicity might be observed. The pattern of results seen after 12 weeks might not continue after many months of exposure. The adduct levels were higher in the rats exposed to B[*a*]P/CB than B[*a*]P after 12 weeks of exposure, so it is possible that this difference might become greater with continued exposure. In addition, the different adduct patterns between the B[*a*]P/CB and B[*a*]P exposures may indicate that other adducts besides the BPDE-DNA adduct are important in potential carcinogenic effects of B[*a*]P/CB exposures. Another factor is the possible influence of a chronic inflammatory response, cell injury, or cell proliferation, all of which accompany long-term exposures to inhaled insoluble particles (Morrow, 1986). Such responses are generally greater after prolonged exposure than in the current 12-week exposure. These responses might be factors in progression to tumors in long-term inhalation exposures of rodents, when large lung burdens of particles

accumulate (Morrow, 1986), and in the increased incidence in tumors when B[*a*]P is merely mixed with Fe_2O_3 particles versus adsorbed onto the particle (Saffiotti et al., 1965).

Studies have also been conducted to evaluate DNA adduct formation in the lungs of animals exposed both to DE and to particles that are nearly devoid of organics (e.g., CB) or that completely lack organic fractions (e.g., TiO_2).

DNA adduct formation in the lungs of animals subjected to long-term exposure to whole DE has been described by Wong et al. (1986). Using tissues from animals of the Mauderly et al. (1987) study, these investigators reported an increase in DNA adduct formation in male and female F344 rats exposed to whole DE (7.1 mg of particles/m^3) for 7 h/day, 5 days/week for up to 30 mo. ^{32}P postlabeling was applied to DNA that was extracted from six control and six exhaust-exposed rats (males and females). Characterization of the adducts and identification of the exhaust components responsible for their formation were not within the scope of the study. The lungs of exhaust-exposed rats were darkly pigmented and contained diesel particle-laden macrophages. Aggregates of these macrophages were frequently associated with alveolar wall fibrosis, bronchiolar metaplasia and, occasionally, squamous metaplasia. Lungs from control rats were not darkly pigmented and had relatively unaltered airways and structures. Autoradiographic analysis revealed elevated levels of DNA adducts in the exhaust-exposed rats. The authors indicated that quantitative and qualitative data regarding DNA adducts resulting from diesel exhaust exposure may be useful for extrapolation to potential effects in humans.

A study by Bond et al. (1989) addressed several key topics regarding the role of DNA adducts in the pulmonary carcinogenicity of DE. Using groups of rats exposed to whole DE at particle concentrations of 0, 0.35, 3.5, 7.0, or 10.0 mg/m^3 for 12 weeks, the relationship between DNA adduct levels and exposure concentration was examined. The data for the exposure levels employed indicated that DNA adduct formation (about 14 adducts per 10^9 bases) was similar across all exposure concentrations and was approximately twice that of the sham-exposed group. The fact that DNA adduct formation was independent of exposure concentration may be explained, in part, by previously reported data (Bond and Mauderly, 1984) showing that metabolism of organics associated with diesel exhaust by the isolated perfused rat lung could be saturated at high concentrations, thereby limiting the production of metabolites required for the formation of DNA adducts.

The time course for DNA adduct formation was also examined by Bond et al. (1989). Over a 12-week period of exposure to diesel exhaust (7 mg/m^3 DPM), lung DNA adducts were found to accumulate slowly. The highest adduct level was reached at 12 weeks, followed by a decline to control level by 4 weeks postexposure. Throughout the exposure period, lung DNA adducts remained constant and at a lower level in sham-exposed rats. The investigators suggested

that the rapid repair of adducts relative to their formation might result in a steady-state level of DNA adducts during long-term exposure.

A dosimetry study examined the distribution of DNA adducts in the respiratory tract to determine if increased DNA adduct formation occurred in regions of the lung where diesel exhaust-induced tumors are formed (Bond et al., 1988). For this study, rats were exposed for 12 weeks to DE at a particle concentration of 10 mg/m^3. DNA adduct levels were highest in peripheral tissue, which is the same region in which tumors occurred in rats in long-term exposure studies (Mauderly et al., 1987). Although these findings suggest that DNA adduct formation and tumor formation are related, the data do not prove the association.

The previous studies provided data regarding the role of DNA adducts in the pulmonary carcinogenesis of DE, but were not designed to provide insight into possible target cells. An additional molecular dosimetry study by Bond et al. (1990a) addressed this topic and also compared the effects of DPM with CB particles that were virtually free of the adsorbed organics found on DPM. In this study, rats were exposed to whole DE (6.2 mg/m^3), CB particles (6.2 mg/m^3 DPM), or clean air 16 h/day, 5 days/week for 12 weeks. Relative to clean air controls, a significant increase in the total DNA adduct level in Type II cells was noted for rats exposed to DE and CB. The exposure to CB and DE resulted in an approximate fourfold increase in adduct level compared with controls. However, the investigators noted that there was a large region of unresolved adducts in the chromatograms from DE-exposed rats and that the total adduct level in these animals may be underestimated. Whether the small amount ($\approx$0.04%) of extractable organics on the CB particles was responsible for the observed DNA adduct formation or the adducts were the result of inflammatory responses to the particles was not determined. This study does, however, demonstrate that Type II cells are possible targets for diesel exhaust exposure.

The report by Bond et al. (1989) provided additional information to suggest a possible relationship between DNA adducts in the lung and DE-induced pulmonary carcinogenesis. For this study, rats, mice, hamsters, and monkeys were exposed to DE at a particle concentration of 8.1 mg/m^3 for 12 weeks. Following this exposure, the levels of lung DNA adducts in rats, a species susceptible to DE-induced carcinogenesis, were shown to be 60% greater than for sham-exposed controls. However, lung DNA adduct levels in mice and hamsters (species that have been shown to be resistant to exhaust-induced carcinogenesis) were very similar to those of respective controls. Also of interest was the finding that DNA adduct levels in the lungs of diesel exhaust-exposed monkeys were 80% greater than those of sham-exposed controls.

In study by Wolff et al. (1990) described earlier in this chapter, levels of DNA adducts as determined by ^{32}P postlabeling were significantly higher for DE-exposed rats than for CB-exposed rats after 12 weeks of exposure to DPM concentrations of 10 mg/m^3. The results of this study suggested that DNA adduct levels are influenced by the organic content of the carbonaceous

particles and that the organic constituents may initiate carcinogenesis. It was hypothesized that the continued inflammatory and proliferative responses may then promote cell transformations. Although the DNA adduct formation may have been the result of very small amounts of organics desorbed from the carbon particles, it is also possible that these adducts are the result of oxygen radicals or other reactive agents released from neutrophils and macrophages. More recent reports have affirmed the latter contention. Randerath et al. (1992) and Williams et al. (1992) provided data showing DNA adduct levels to be increased following exposures to CB and that no organic fraction was involved.

Gallagher et al. (1994) noted that DNA adduct-like compounds were formed in rat lungs following exposures to DE, TiO_2, or CB, but that the total adduct levels were not significantly elevated by DE exposure. Exposure to DE resulted in DNA adducts, possibly from nitro-PAHs associated with the organic fraction of the DPM, but the overall significance of this mechanism in the carcinogenic response is uncertain.

The uncertainty of the role of a genotoxic mechanism was further shown by Swafford et al. (1995), who detected no differences in mutational patterns in CB-induced or diesel exhaust-induced pulmonary carcinomas. It was, however, noted that inactivation of the *p53* may have a role in neoplastic responses with a squamous-cell carcinoma component.

Based on the assumption that DNA adduct formation is a critical step in the initiation of carcinogenesis (Harris, 1985), increased residence time of PAHs in the lung would increase the opportunity for metabolism and subsequent adduct formation. Some involvement of the organic components is suggested by data showing the formation of DNA adducts in exposed animals and by the known carcinogenic and mutagenic potential of many of the compounds in diesel exhaust. Several studies affirm the bioavailability from inhaled diesel exhaust particles of compounds such as B[*a*]P and 1-NP, which are known to be carcinogenic or mutagenic. Furthermore, the fact that xenobiotics may undergo biotransformation to reactive intermediates following their entry into the body via inhalation of DPM has been demonstrated for B[*a*]P and various nitroarenes. However, results from the metabolism/disposition studies using carbon particles to which organics have been experimentally adsorbed must be interpreted with caution. The concentration of organics on these particles is probably much greater than the monomolecular or bimolecular layer on actual DPM and, therefore, might facilitate desorption of the organics from these experimentally prepared particles.

The role of DNA adducts in DE-induced pulmonary carcinogenesis requires further investigation (Bond, 1993). Because DNA adduct formation is observed after exposure to CB particles, formation of these adducts by particle-adsorbed organics does not appear to be a definitive mechanism for explaining the carcinogenic response observed in rats. However, a possible (albeit minor) contributory role for particle-adsorbed organics cannot be totally

dismissed. Higher concentrations of CB than DPM were required to induce adduct formation, and Gallagher et al. (1994) reported a nuclease-sensitive adduct in rats exposed to DPM but not CB. It was hypothesized that this adduct may have resulted from exposure to nitro-PAHs.

Several investigators have reported on DNA adducts in humans exposed to diesel exhaust. In studying biomarkers of exposure, distinct adduct patterns were found among garage workers occupationally exposed to diesel exhaust when compared to nonexposed controls (Nielsen and Autrup, 1994). Furthermore, the findings were concordant with the adduct patterns observed in groups exposed to low concentrations of PAHs from combustion processes. Hemminki et al. (1994) also reported elevated levels of DNA adducts in lymphocytes from garage workers with known diesel exhaust exposure compared to nonexposed mechanics. The adduct levels (up to 3.63 adducts/10^8 nucleotides) were significantly greater in diesel exhaust-exposed groups when compared to nonexposed groups. Hou et al. (1995) found elevated adduct levels in bus maintenance workers exposed to diesel exhaust. Although no difference in mutant frequency was observed between the groups, the adduct levels were significantly different (3.2 vs. 2.3×10^{-8}). Nielsen et al. (1996) measured three biomarkers in DE-exposed bus garage workers: lymphocyte DNA adducts, hydroxyethylvaline adducts in hemoglobin, and 1-hydroxypyrene in urine. Significantly increased levels were reported for all three. Qu et al. (1996) detected increased adduct levels, as well as increases in some individual adducts, in the blood of underground coal miners exposed to DE.

10.5. SUMMARY OF METABOLISM AND MECHANISM OF ACTION OF CARCINOGENIC COMPONENTS OF DIESEL EXHAUST

Recent studies have shown tumor rates resulting from exposures to nearly organic-free CB particles to be similar to those observed for DE exposures, thus providing strong evidence for an epigenetic mechanism underlying DE-induced pulmonary carcinogenesis in rats at high doses. A nongenotoxic mechanism is also supported by the fact that carbon particles per se cause inflammatory responses and increased epithelial cell proliferation and that AM function may be compromised under conditions of particle overload. A mechanism was proposed in which particle-overloaded phagocytic cells secreted a variety of inflammatory mediators, stimulating cell proliferation and increasing the likelihood that any oxidant-induced or spontaneously occurring genetic damage would become fixed and clonally expanded, resulting in an increased probability of neoplastic change. The development of lung tumors in rats following chronic exposures to diesel exhaust was detected under conditions in which AM-mediated particle clearance from the lung provides support for this hypothesis. It should be noted, however, that the bioassays lacked sensitivity to detect tumors at nonoverload conditions.

It is generally accepted that one of the underlying mechanisms of carcinogenesis involves the formation of covalent adducts with DNA, resulting in the alteration of cellular genetic information. Several reports have provided data indicating that such adducts are formed in animals after long-term exposure to DE. The premise that DNA adduct formation plays a role in DE-induced carcinogenesis is substantiated by several findings, including an increase in DNA adducts in the same pulmonary regions where tumors occur and higher DNA adduct levels in species known to be susceptible to DE-induced tumors. However, the lack of an exposure response for DNA adduct formation, as demonstrated by the molecular dosimetry studies reported by Bond et al. (1990b), suggests the involvement of additional mechanisms.

While particle overload mechanisms are likely to predominate at high exposure concentrations, organics are likely to play a greater role for any effects that may occur at non-overload exposure conditions. Although the lung's AMs, which phagocytize deposited DPM, may participate in the gradual in situ extraction and metabolism of procarcinogens associated with the diesel particles, it is uncertain if the mutagenic organics are or can be eluted in sufficient amounts to be relevant to a carcinogenic response in the rat model. The much slower particle clearance rates in humans, however, allow for greater efficiency of extraction. While both DE and CB have been reported to increase DNA adducts to a similar degree, DE is effective in non-particle-overload conditions, suggesting again that the organics may play a role in tumor induction. The participation of organics is supported by reports of increased DNA adducts in humans occupationally exposed to diesel exhaust (see Section 10.4).

Low-dose effects of DPM must also be considered, including the findings of Riebe-Imre et al. (1994), who showed that CB may induce chromosomal damage and cytoskeletal alterations in vitro at doses that are not cytotoxic. The role of reactive oxygen species must also be considered relative to low-dose DE exposure. The normal tumoricidal activities of the AMs may be compromised upon interaction with excessive numbers of diesel particles, and diesel particle-macrophage interactions could lead to the generation of reactive oxygen species that have been shown to be at least mutagenic. The in vitro formation of active oxygen radicals by DE exposure reported by Sagai et al. (1993) indicated that reactive oxygen species could be formed without bioactivation, and may thus occur even under nonoverload conditions. The in vivo induction of oxygen radical-induced DNA damage (Nagashima et al., 1995) provided additional support for low-dose particle effects.

Caution must be exercised in extrapolating observations made in animal models to humans when assessing the potential for DE-induced pulmonary carcinogenesis. The carcinogenic response and the formation of DNA adducts in rats exposed to diesel exhaust and other particles at high exposure concentrations may be species-specific and not particle-specific. However, DNA

adduct data and the possible involvement of reactive oxygen species may be relevant in long-term occupational exposure of humans to low concentrations.

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